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ASSESSMENT OF FIRST GENERATION ANDROGENIC RICE LINES FOR TRUE DOUBLED HAPLOIDS

LOVELIN KAUSHAL[1], SM BALACHANDRAN[2], K ULAGANATHAN[3] & VINAY SHENOY[4]

[1,4]Barwale Foundation, Himayathnagar, Hyderabad, Andhra Pradesh, India

[2]Directorate of Rice Research (DRR), Hyderabad, Telangana, India

[3]Centre for Plant Molecular Biology (CPMB), Osmania University, Hyderabad, Telangana, India

ABSTRACT

Doubled haploidy is a fundamental tool in plant breeding as it provides the fastest way to generate populations of meiotic recombinants in a genetically fixed state. The homogeneity and variability of androgenic lines from 5 different populations has been evaluated to confirm the first generation anther derived rice line for true doubled haploids. The DH lines were uniform as expected. The homogeneity of the doubled haploid (DH) lines was shown by the absence of heterozygosity at molecular level and absence of segregation for morphological traits. This study confirmed the first generation DH lines are indeed true doubled haploids.

KEYWORDS: Doubled Haploidy, Doubled Haploid, Uniformity, Variability

INTRODUCTION

Rice is one of the most important cereals, providing carbohydrate sources for over half of the world's population (Cassman, 1999; Khush, 2005). To meet the demand of increasing population and maintain self sufficiency the present rice production needs to be increased by 30% by the year 2020 (Hossian, 1997). Hybrid rice technology offers a potentially viable option for increasing rice yield beyond the level of high yielding varieties, as hybrid rice has about 15-20% yield advantage over the conventional pure line varieties (Yuan, 1994). For development of commercial hybrids, choice of appropriate parental lines possessing good combining ability, high yield potential, good grain quality, tolerance or resistance to abiotic and biotic factor is a prerequisite.

Rice breeder used different method to develop improved and pure hybrid rice parental line. Isolation of homozygous and homogeneous parental line through conventional breeding requires several cycles of in breeding and selection making it the most tedious, time consuming, and expensive phase of any breeding program. Furthermore, in conventional breeding, isolation of truly homozygous line is rare and most selections contain some heterozygous loci (Snape, 1989; Raina, 1997; Baenziger et al., 2006). Recent advances in plant tissue culture and its related disciplines opened an avenue that greatly facilitated the doubled haploidy breeding scheme, and this enables the extraction of instant homozygous line in a single generation (Baenzier et al., 1989; Wu et al., 2012).

Doubled haploidy (DH) lines are routinely applied in many commercial hybrid breeding program such as maize, wheat and rice. Major advantages of DH lines compared to selfed lines include, (i) maximum genetic variance between lines for per se and test cross performance from the first generation (ii) reduced breeding length, (iii) perfect fulfilment of DUS (distinction, uniformity, stability) criteria for variety protection, (iv) reduced expenses of selfing and maintenance

breeding, (v) simplified logistics, and (vi) increased efficiency in marker assisted selection, gene introgression, and stacking genes in lines (Geiger and Gordillo, 2009). More than 280 varieties have been produced with the use of doubled haploidy in several crops (http://www.scri.sari.ac.uk/assoc/COST851/COSThome.htm), with majority of the protocols referred to as anther culture (Germana, 2012). Even in the case of rice, more than 20 rice varieties obtained through DH production worldwide (Zepata-Arias, 2003). Despite the practical use of the technique in rice breeding, there still a limited understanding of the potential for cultivar development via anther culture because of its inherent factors, such as genotypic dependence of androgenesis (Kaushal et al., 2014a; 2014b; Kaushal et al., 2015), the deleterious effect of somoclonal variation (Onoo, 1983), distortion in segregation by gametic selection during androgenesis (Murigneux et al., 1993) and only once chance of recombination before fixation in the F_1 system (Snape, 1976). These factors can influence genetic variation and creations of desirable recombinants in the breeding lines derived from anther culture.

Doubled haploidy allows early expression of not only dominant but recessive genes (Sasmita, 2009) and increases the selection efficiency as the number of plants required to obtain the desired recombinants are less than the conventional breeding (Torrizo and Zapata, 1986). Zhang (1989) reported that trait of doubled haploid plants of same lines was uniform and remains stable from generation to generation and selection can be done directly on the early generation plants. Previous investigation by Herawati et al. (2008) recognized the first generation anther derived line as pure line and released 58 first generation upland dihaploid. Combined DH and molecular marker technologies can further enhance genetic gain, facilitate multiple trait stacking/pyramiding, increases efficiency and probability of successful product development as molecular marker could confirm or deny the level of homozygosity of DH populations. Among the most commonly used molecular markers, simple sequence repeats (SSRs) are efficient, cost effective and powerful in the assessment of uniformity and variability (Ravi et al., 2003). SSR analysis was successfully used for evaluation of genetic variability and similarity among rice (Olufowote et al., 1997), wheat (Nasab et al., 2013) and Barley (Spunarova et al., 2005).

Since the cellular origin of the plants produced is not always certain, screening techniques are needed to validate that the anther derived DH plants are indeed homozygous and distinct from each other. Thus, the objective of the current investigation to assess the first generation spontaneously developed androgenic line for true doubled haploids. The molecular analysis could provide information on the cellular origin (somatic or gametic) of all tested DHLs.

MATERIALS AND METHODS

Materials

249 doubled haploid rice lines derived through *in vitro* anther culture of F_1 crossing between five Sub-1 donors with recurrent parent (IR58025eB), improved hybrid rice parental with *eui* gene (Table 1). The populations were generated at Barwale foundation.

Table 1: Size of Populations Studied

Code	Cross	F_1	No DHLs
SA	IR58025eB x Samba Mahsuri Sub-1	F_1 with *eui* x *Sub-1*genes	31
SB	IR58025eB x IR-64 Sub-1	„	14
SC	IR58025eB x BR-11 Sub-1	„	47
SD	IR58025eB x TDK-1 Sub-1	„	48
SE	IR58025eB x Swarna Sub-1	„	109

MORPHOLOGICAL EVALUATIONS

Completely randomized design was used with 2 replications with 15 cm x 20 cm spacing. In each replication average of three plants were undertaken for observation and data analysis. Nine morpho-agronomic traits were taken into consideration for the investigation (Table 2).

Table 2: List of Traits and Sample Size for these Measurements

Traits	Abbreviation
Plant height (cm)	PH
No of panicles per plant	PN
Total panicle length (cm)	TPL
Leaf length (cm)	LL
Leaf width (cm)	LW
Internode III (cm)	INT III
Internode II (cm)	INT II
Internode I (cm)	INT I
Exertion of panicle (cm)	EXE

MOLECULAR ANALYSIS

Molecular analysis was done at DH_0 generation. Total genomic DNA was extracted from the tested androgenic plants. Leaves tissue of 2 cm length was ground in 800 µl extraction buffer and incubated at 65° C in a water bath for 45 minutes and extracted with 400 µl Chloroform and Iso-amyl alcohol (24:1) mixture. Supernatant was taken in another eppendorf tube and the DNA was precipitated with 2/3 volumes of absolute alcohol, incubated for 20 minutes at -20° C and centrifuged for 15 min, further pellet was washed with 70% ethanol twice and dried. Further, the DNA pellet was resuspended in 100µl of TE buffer and kept in 4° C for future use.

To confirm the molecular uniformity, each DH populations was screened with two polymorphic microsatellite primers pairs (SA with RM 16 and RM 234, SB with RM 234 and RM 3620, SC with RM 17506 and RM 340, SD with RM 17506 and RM 228 and SE with RM 17506 and RM 6297, respectively). Further, RM 5970 was used to confirm the presence of *eui* gene in DH_0 plants. The amplification mixture consisted of 15 µl reaction mixture containing 1.5 ng DNA, 200 µM of each dNTPs, 1 µM each of forward and reverse primers, 1 unit Taq polymerase. After denaturing the genomic DNA template at 95° C for 5 min, PCR was performed with 35 cycles of denaturing at 95° C for 45 sec, annealing at 58° C for 45 sec, extension at 72° C for 1 min, and final extension incubation at 72° C for 10 minutes.

PCR amplified products were separated in 8% CBS PAGE at 120 V for 2 h in 0.5 x TBE buffer and stained with florescent ethidium bromide. Picture of the ethidium bromide–stained amplified PCR product was taken with a camera (UV-gel documentation system).

STATISTICAL ANALYSIS

The phenotypic data generated were statistically analyzed to assess the intraline uniformity as well as the extent of the variability among the DHLs employing Crop Stat Version 7.2.2007.3 (IRRI, 2007). The trait homogeneity and variability was determined based on the value of variance (σ2), standard deviation of mean (σ) and coefficient of variation

(CV %). Scattered plot analysis and correlation study was done using Microsoft Excel program. Each SSR marker was scored as presence of the band (1 for P1, 2 for P2, 3 for heterozygous band and 4 for neither type). Each band was regarded as a locus.

RESULTS

Analysis of variance (ANOVA) revealed a highly significant genotypic difference ($p<0.001$) among all DHLs. The ANOVA main table is depicted in Table 3. The ANOVA results indicated that there were highly significant differences in all studied traits among 5 different DH populations. The trait homogeneity and variability was observed from the present investigation (Figures 1-4). The trait variability among the 5 population are depicted in Table 4(a-e). Maximum variance was observed for PH ranged from 116.17 for SA to 437.77 for SC. For trait EXE the variance value ranged from 16.95 for SB to 107.52 for SE. However, minimum variance was identified for LW across all 5 populations (0.003 for SB to 0.15 for SD). SD of mean, another reliable parameter to measure trait variability and uniformity of a character was found to be more uniform for LW across 5 different populations in comparison to other characters as evident from their low SD and narrow range of variation (0.13 in SE to 0.23 in SA). However, SD of mean was found to be high for PH across the investigated population ranging from 13.91 for SD to 20.92 for SC, followed by EXE ranged from 2.63 in SB to 6.23 in SA. This implies that high variability was found for PH followed by EXE. Furthermore, variation of coefficient was low and did not exceed 17.05 %. For EXE, CV % ranged from 5.34% in SE to 17.05% in SB, followed by PN (6.06 % for SB to 8.76% for SE) and INT III (5.97% for SA to 7.36 % for SE). Whereas, CV % was very low for PH, ranging from 0.88 to for SC to 2.97 for SE.

Table 3: ANOVA for Trait Uniformity/Variability

	SA[a]		SB		SC		SD		SE	
Source of Variation	**F Value**	**CV%**	**F Value**	**CV%**	**F Value**	**CV%**	**F Value**	**CV%**	**F Value**	**CV%**
PH[b]	287.52*	1.22	341.4*	0.93	655.36*	0.88	247.26*	1.03	80.75*	2.97
PN	6.44*	6.67	4.11*	6.06	5.17*	8.55	3.45*	8.64	4.53*	8.76
TPL	35.88*	2.11	16.77*	2.4	36.4*	2.14	38.78*	2.05	34.73*	2.36
LL	13.63*	2.32	2.53**	3.37	34.35*	3	25.94*	2.38	16.2*	2.18
LW	27.14*	3.66	5.5*	6.04	8.9*	5.63	6.73*	4.89	7.98*	4.45
INT I	8.06*	5.97	7.42*	6.68	13.65*	7.19	14.42*	6.13	30*	7.36
INT II	34.24*	3.06	25.31*	3	39.37*	2.49	21.36*	2.66	43.19*	3.27
INT I	22.55*	1.63	14.73*	1.71	11.44*	3.09	12.42*	2.36	26.22*	2.35
EXE	361.53*	6.98	10.22*	17.05	226.28*	6.03	168.66*	9.11	356.07*	5.34

a: SA to SE as in Table 1; b: PH to EXE as in Table 2

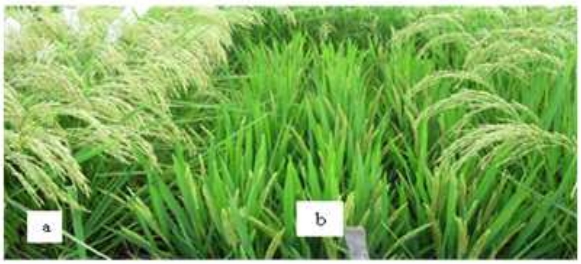


Figure1:Intraline Uniformity of DH_1 Line
a.Non *eui* DH line b.*eui* DH Line

Figure 2: Intraline Variability of DH_1
a.Non *eui* DH Line b.*eui* DH Line

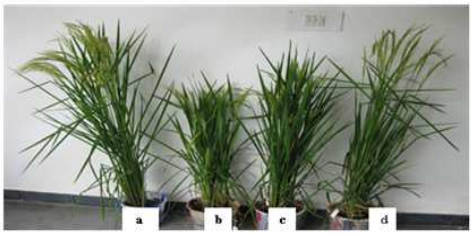


Figure 3: Variation on DH Lines Developed a.IR58025eB, b. Swarna Sub1 c. Non *EUI d. EUI* Plant

Figure 4:Expression of *EUI* Trait a. IR58025eB b. Non EUI Panicle c. *EUI* Panicle d. Samba Mahsuri Sub 1

Table 4: Variability among DHLs for Agronomic Traits

(a) SA DHLs

SC	Variability in R1							Variability in R2						
Trait	F1	Min	Max	Range	Mean	Variance	SD	F1	Min	Max	Range	Mean	Variance	SD
PH	121.00	76.00	159.00	83.00	130.10	437.77	20.92	121.50	77.00	158.33	81.33	130.27	431.16	20.76
PN	10.50	7.33	13.00	5.67	10.03	2.51	1.58	12.00	6.33	13.00	6.67	9.88	1.97	1.40
TPL	27.50	19.83	31.50	11.67	26.84	6.02	2.45	28.12	21.00	32.67	11.67	26.94	6.39	2.53
LL	31.00	23.67	45.67	22.00	32.96	17.66	4.20	30.00	24.33	45.00	20.67	33.01	16.88	4.11
LW	1.85	1.23	2.00	0.77	1.64	0.05	0.21	2.00	1.40	2.00	0.60	1.65	0.04	0.20
INT III	10.00	7.33	18.67	11.34	12.86	6.25	2.50	9.75	8.50	19.33	10.83	12.56	5.96	2.44
INT II	23.25	18.17	30.50	12.33	27.41	10.27	3.21	26.25	18.67	30.17	11.50	27.65	8.78	2.96
INT I	32.25	29.33	39.33	10.00	34.36	7.39	2.72	30.25	26.50	39.00	12.50	34.24	6.56	2.56
EXE	+2.75	-3.67	14.50	18.17	7.85	25.49	5.05	+5.75	-3.83	13.83	17.66	8.00	26.45	5.14

(b) SB DHLs

SB	Variability in R1							Variability in R2						
Trait	F1	Min	Max	Range	Mean	Variance	SD	F1	Min	Max	Range	Mean	Variance	SD
PH	111.50	91.67	132	42.33	118.07	213.03	14.17	114.50	91.67	134	40.33	117.97	200.82	14.17
PN	9.00	9	13	3.67	11.14	1.14	1.08	11.50	10	13.67	4.00	10.99	1.16	1.07
TPL	26.75	22.5	29.5	7.25	25.92	3.60	1.81	27.50	22.33	29.58	7.00	26.04	3.28	1.81
LL	29.50	29	32.67	5.33	30.90	2.77	1.00	29.50	29	34.33	3.67	30.49	1.00	1.00
LW	2.00	1.33	1.9	0.70	1.54	0.03	0.15	2.00	1.2	1.9	0.57	1.56	0.02	0.14
INT III	10.00	9	13.67	5.83	11.21	2.73	1.46	9.75	9	14.83	4.67	11.52	2.12	1.45
INT II	26.00	20.17	29.83	10.00	27.11	10.15	2.71	26.25	19.67	29.67	9.66	27.26	7.36	2.71
INT I	30.75	30.83	35.17	5.33	33.65	3.22	1.39	30.25	30.17	35.5	4.34	33.25	1.94	1.39
EXE	-4.50	-3.67	11	14.84	8.26	16.95	2.63	5.75	-3.67	11.17	7.33	8.86	6.92	2.63

(c) SC DHLs

SC	Variability in R1							Variability in R2						
Trait	F1	Min	Max	Range	Mean	Variance	SD	F1	Min	Max	Range	Mean	Variance	SD
PH	121.00	76.00	159.00	83.00	130.10	437.77	20.92	121.50	77.00	158.33	81.33	130.27	431.16	20.76
PN	10.50	7.33	13.00	5.67	10.03	2.51	1.58	12.00	6.33	13.00	6.67	9.88	1.97	1.40
TPL	27.50	19.83	31.50	11.67	26.84	6.02	2.45	28.12	21.00	32.67	11.67	26.94	6.39	2.53
LL	31.00	23.67	45.67	22.00	32.96	17.66	4.20	30.00	24.33	45.00	20.67	33.01	16.88	4.11
LW	1.85	1.23	2.00	0.77	1.64	0.05	0.21	2.00	1.40	2.00	0.60	1.65	0.04	0.20
INT III	10.00	7.33	18.67	11.34	12.86	6.25	2.50	9.75	8.50	19.33	10.83	12.56	5.96	2.44
INT II	23.25	18.17	30.50	12.33	27.41	10.27	3.21	26.25	18.67	30.17	11.50	27.65	8.78	2.96
INT I	32.25	29.33	39.33	10.00	34.36	7.39	2.72	30.25	26.50	39.00	12.50	34.24	6.56	2.56
EXE	+2.75	-3.67	14.50	18.17	7.85	25.49	5.05	+5.75	-3.83	13.83	17.66	8.00	26.45	5.14

(d) SD DHLs

SD	Variability in R1							Variability in R2						
Trait	F1	Min	Max	Range	Mean	Variance	SD	F1	Min	Max	Range	Mean	Variance	SD
PH	109.50	86.33	149.33	63.00	119.30	193.42	13.91	109.50	88.33	144.33	56.00	119.80	185.23	13.61
PN	11.50	8.67	14.33	5.66	10.54	1.63	1.28	11.00	8.00	13.67	5.67	10.56	2.07	1.44
TPL	26.12	20.83	30.00	9.17	25.94	5.58	2.36	25.75	20.17	30.00	9.83	26.18	5.72	2.39
LL	30.00	23.67	45.00	21.33	30.56	8.32	2.88	31.00	24.67	42.33	17.66	30.51	5.86	2.42
LW	1.60	1.20	1.93	0.73	1.48	0.02	0.15	1.50	1.23	1.97	0.74	1.53	0.02	0.14
INT III	11.50	7.00	15.50	8.50	11.19	3.88	1.97	9.50	7.17	14.83	7.66	11.27	3.44	1.86
INT II	21.75	19.17	29.67	10.50	26.68	6.20	2.49	21.00	20.00	29.50	9.50	26.91	5.12	2.26
INT I	32.25	29.83	37.00	7.17	33.63	3.42	1.85	32.75	28.33	38.00	9.67	33.58	3.51	1.87
EXE	-2.50	-4.50	11.00	15.50	5.87	24.56	4.96	-2.50	-4.17	10.83	15.00	6.10	25.86	5.08

(e) SE DHLs

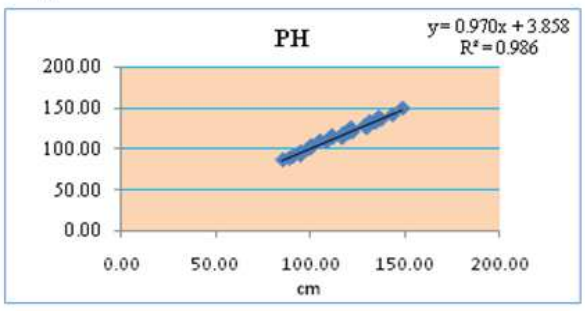
SE	Variability in R1							Variability in R2						
Trait	F1	Min	Max	Range	Mean	Variance	SD	F1	Min	Max	Range	Mean	Variance	SD
PH	102.50	70.67	151.67	81.00	112.95	459.13	21.43	106.00	71.00	150.33	79.33	112.55	458.25	21.41
PN	12.00	7.33	13.33	6.00	10.18	2.05	1.43	10.50	6.00	14.33	8.33	10.16	2.34	1.53
TPL	25.75	18.50	29.75	11.25	25.22	6.47	2.54	25.75	19.00	30.00	11.00	25.43	6.33	2.52
LL	29.00	22.00	33.33	11.33	28.89	3.74	1.93	30.00	22.67	33.33	10.66	29.33	3.18	1.78
LW	1.90	1.13	2.00	0.87	1.46	0.02	0.14	1.95	1.17	2.00	0.83	1.48	0.02	0.13
INT III	15.75	4.83	16.66	95.17	10.91	82.64	9.09	10.75	4.50	16.17	11.67	10.16	8.73	2.96
INT II	22.00	16.17	32.00	83.83	25.94	67.21	8.20	21.00	17.00	31.00	14.00	25.66	14.56	3.82
INT I	30.50	24.17	38.16	75.83	33.04	49.67	7.05	30.75	25.50	39.67	14.17	32.67	8.10	2.85
EXE	-2.25	-4.83	13.50	104.83	8.43	107.52	10.37	-3.00	-5.00	14.00	19.00	7.72	30.56	5.53

In scattered plot analysis, a significant and high r^2 value was obtained for most of the traits, except PN with r^2 value of 0.53 (Table 5). The scattered plot representation of SA population is presented in Figure 5(a-c). Further, the result of the correlation analysis as shown by their coefficient of correlations Table 6(a-e). A strong and positive correlation was observed between PH with other traits, such as TPL (0.48-0.85), INT III (0.58-0.87), INT II (0.73-0.98), INT I (0.73-0.95) and EXE (0.69 to 0.84) among five populations. However, PH was negative to positive, but weak in association to PN (-0.57 to +0.07) and negative to LW (-0.42 to -0.10). TPL was moderate and positively co-relate with INT II, INT I and EXE with r values ranging 0.40-0.54 for INT III, 0.03-0.74 for INT II, 0.59-0.79 for INT I and 0.42-0.74 for EXE. Again INT III was moderate and positive in relation to INT II (0.51-0.84), INT I (0.52-0.79) and EXE (0.27-0.65). Similarly, INT II was positively correlated with INT I (0.67-0.90) and EXE (0.65-0.88). Furthermore, INT I was positively correlated with EXE (0.66-0.81). However, LW is negative to positive, but weak in relation to other traits.

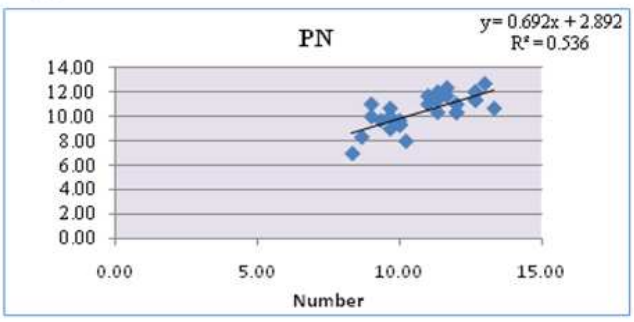
Table 5: Scattered Plot Analysis

Traits	r^2 Value				
	SA	SB	SC	SD	SE
H	0.98	0.98	0.99	0.98	0.95
PN	0.53	0.37	0.46	0.37	0.40
TPL	0.90	0.78	0.89	0.9	0.89
LL	0.81	0.78	0.89	0.88	0.78
LW	0.88	0.61	0.64	0.54	0.6
INT III	0.83	0.59	0.74	0.76	0.83
INT II	0.91	0.87	0.90	0.83	0.91
INT I	0.61	0.81	0.70	0.72	0.85
EXE	0.98	0.81	0.98	0.97	0.98

(a)



(b)



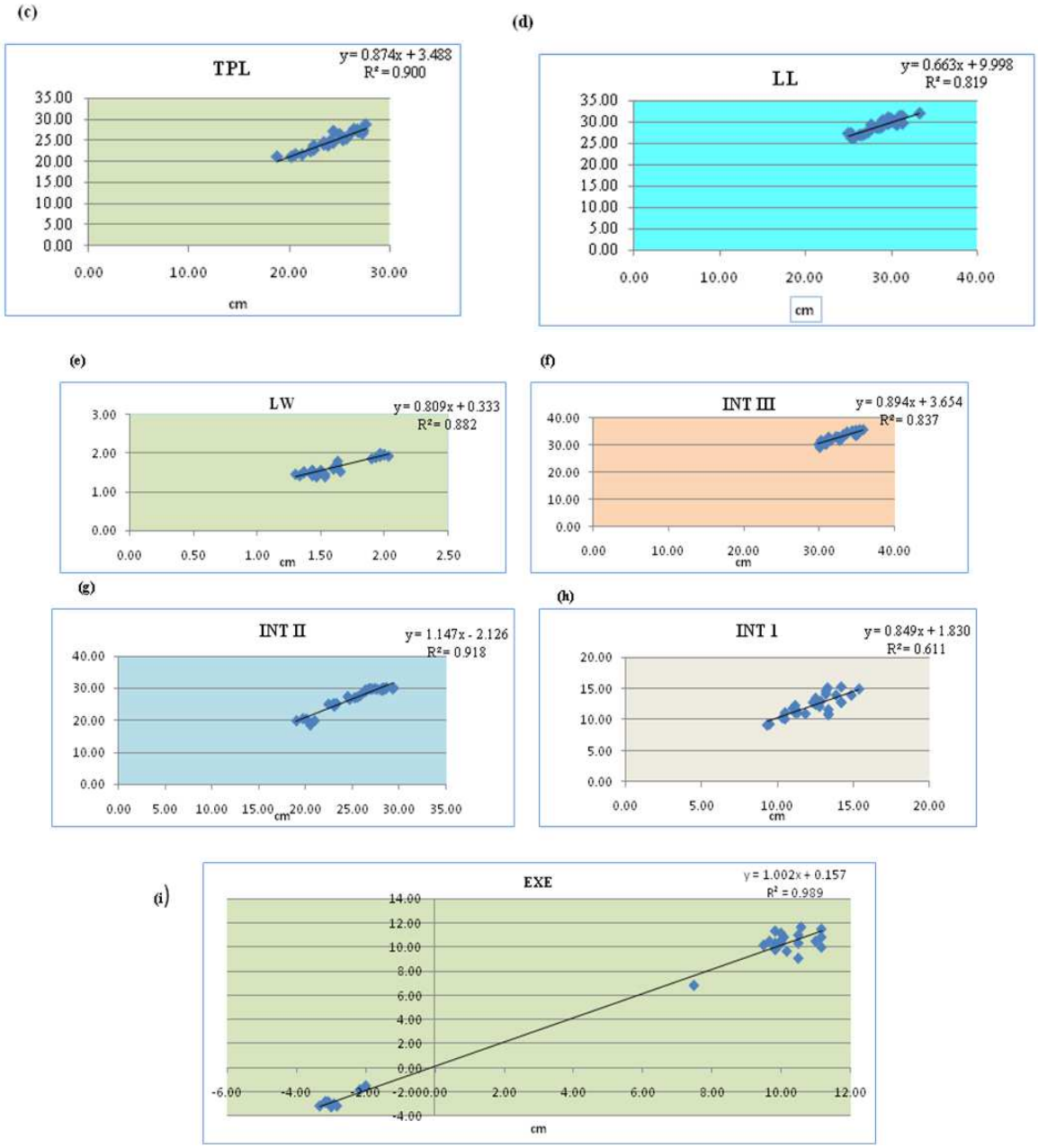


Figure 5: (a-i) Scattered Plot Analysis of SA DHLs

Table 6: (a-e) Correlation Analyses

(a)

SA	PH	PN	TPL	LL	LW	INT III	INT II	INT I	EXE
PH	1.00								
PN	-0.24	1.00							
TPL	0.85	-0.38	1.00						
LL	0.36	-0.31	0.45	1.00					
LW	-0.20	0.06	-0.21	0.00	1.00				
INT III	0.58	-0.25	0.40	0.52	0.05	1.00			
INT II	0.98	-0.20	0.84	0.36	-0.23	0.61	1.00		
INT I	0.88	-0.30	0.79	0.45	-0.01	0.52	0.88	1.00	
EXE	0.84	-0.12	0.74	0.06	-0.31	0.27	0.88	0.71	1.00
EXE	0.82	-0.41	0.46	0.03	-0.52	0.51	0.65	0.81	1.00

(b)

SB	PH	PN	TPL	LL	LW	INT III	INT II	INT I	EXE
PH	1								
PN	-0.57	1.00							
TPL	0.48	-0.03	1.00						
LL	-0.26	0.31	0.21	1.00					
LW	-0.42	0.41	0.13	0.40	1.00				
INT III	0.74	-0.49	0.40	-0.33	-0.03	1.00			
INT II	0.84	-0.47	0.03	-0.44	-0.64	0.51	1.00		
INT I	0.95	-0.37	0.59	-0.27	-0.37	0.73	0.74	1.00	
EXE	0.82	-0.41	0.46	0.03	-0.52	0.51	0.65	0.81	1.00

(C)

SC	PH	PN	TPL	LL	LW	INT III	INT II	INT I	EXE
PH	1								
PN	-0.57	1.00							
TPL	0.77	-0.52	1.00						
LL	0.37	-0.40	0.56	1.00					
LW	-0.21	-0.12	-0.11	-0.03	1.00				
INT III	0.75	-0.53	0.44	0.29	0.02	1.00			
INT II	0.82	-0.42	0.74	0.41	-0.27	0.64	1.00		
INT I	0.73	-0.54	0.74	0.72	-0.04	0.62	0.67	1.00	
EXE	0.69	-0.32	0.64	0.42	-0.32	0.46	0.73	0.66	1

(d)

SD	PH	PN	TPL	LL	LW	INT III	INT II	INT I	EXE
PH	1								
PN	0.07	1.00							
TPL	0.70	0.12	1.00						
LL	0.50	0.11	0.59	1.00					
LW	-0.10	-0.26	0.08	0.17	1.00				
INT III	0.80	0.05	0.54	0.35	0.09	1.00			
INT II	0.73	0.17	0.44	0.30	-0.11	0.72	1.00		
INT I	0.82	0.09	0.74	0.34	-0.11	0.69	0.68	1.00	
EXE	0.75	0.20	0.54	0.31	-0.37	0.48	0.64	0.77	1

(e)

SE	PH	PN	TPL	LL	LW	INT III	INT II	INT I	EXE
PH	1.00								
PN	-0.24	1.00							
TPL	0.70	-0.34	1.00						
LL	0.27	-0.08	0.52	1.00					
LW	-0.12	0.21	-0.13	0.15	1.00				
INT III	0.87	-0.19	0.51	0.14	-0.05	1.00			
INT II	0.92	-0.19	0.61	0.24	-0.06	0.84	1.00		
INT I	0.90	-0.31	0.75	0.43	-0.07	0.79	0.90	1.00	
EXE	0.82	-0.12	0.46	0.00	-0.18	0.65	0.80	0.72	1.00

Table 7: Phenotypic Characterization of *EUI* /non *EUI* DHLs

Code	SA		SB		SC		SD		SE	
DHLs	*EUI*	Non *EUI*	*EUI*	Non *EUI*	*EUI*	Non *EUI*	*EUI*	Non *EUI*	*EUI*	Non *EUI*
PH	108.33 - 148.67	85.55-105	112-134	91.67-94.67	109.33-157.33	76-159	88.33-149.33	86.33-123.33	80.67-151.66	70.67-125-33
PN	8.33-13	9-13.33	10-12.33	113.-13.67	7.33-13	8.00-13	8-14.33	8.67-12	7.66-13.33	7.33-13
TPL	22.50-27.67	18.83-24.50	23-29.58	22.50-26.17	23.50-30	19.83-31.50	20.17-30	20.83-29.33	20.50-29.75	18.50-28.58
LL	25-33.33	25-31.33	29.33-34.33	29-32.67	23.67-45.67	26.67-37.33	24.67-45	23.67-32.33	22-33	23-33.33
LW	1.3-2.03	1.43-2	1.20-1.73	1.63-1.90	1.23-2	1.50-2	1.20-1.93	1.27-1.73	1.13-1.8	1.23-2.00
INT III	10.50-15.33	9.33-14.17	9.83-14.83	9-9.83	9.33-18.67	7.33-15.17	7-15.50	7.67-13.33	5.33-16.66	4.83-12
INT II	24.44-29.35	18.98-23.25	26.33-29.67	19.67-25.33	24-30.50	18.17-28.67	20-29.67	19.17-28.83	19-32	16.16-26.66
INT I	30.17-35.83	29.83-32.67	32.67-35.50	30.17-31.67	29.50-39.33	29.33-36.50	28.33-37	29.83-35.67	25.16-38-16	24.16-34.16
EXE	(+7.48)-(+11.17)	(-3.33)-(-2)	(+9)-(+11.17)	(-3.67)-(+4.17)	(+7.25)-(+14.50)	(-3.67)-(+4.83)	(+7.17)-(+11)	(-4.17)-(+5.67)	(+6.86)-(+13.5)	(-4.83)-(+5.53)

The number of alleles detected by each marker varied from 1 to 3. Of the 249 DHLs, 247 androgenic plants showed either parental banding pattern (Figure 6). However, 2 DHLs showed the presence of the both parental bands of similar intensities indicating heterozygosity. Further, the foreground screening with RM 5970 identified a total of 152 plants with *eui* positive, whereas only 71 were with absence of *eui* gene (Figure 7). The Results of foreground selection is presented in Table 8.

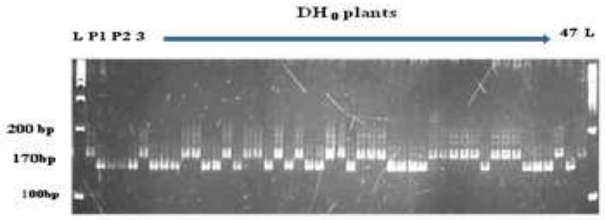


Figure 6: Molecular unifermity screening DH_1 Plants using RM 374 L=Ladder (100bp) P1=IR58025eB, P2= samba Mahusri sub-1 3-47=SA DH_1 plants

Figure 7: foreground screening using RM 5890primer l=Ladder (100bp), P1=IR58025eB, P2=BR 11sub-1 3-31=sc DH_1 plants

Table 8: Segregation of *eui* gene

Code	No of DH Lines Screened	*eui* Positive	*eui* Negative
SA	31	22	9
SB	14	11	3
SC	47	26	12
SD	48	20	16
SE	109	73	31
Total	**249**	**152**	**71**

DISCUSSIONS

The intraline uniformity and interline variability of agro-morphogenic traits are very essential to use the prominent lines as a variety or parental lines. The observations of the DH lines under present investigation revealed the high homogeneity of the material derived from anther culture *in vitro*. The analysis of variance ($p<0.001$) revealed that an individual line within the population was different from each other indicating the origin of DHLs from individual pollen. Further, the progenies of a single line showed uniform morphological traits, reflect the intraline uniformity of DH line in the study. These results also supports the previous reports indicating that each individual plant in a doubled haploid line have a uniform agronomic traits (Sasmita, 2009; Suhartini and Somantri, 2000). Intraline uniformity is also an indication of

pollen origin of DH lines and not from anther wall. Our result is consistent with Zhang's investigation (1989) which indicates that all the traits of doubled haploid lines originated from pollen, not from anther wall.

The high variance and SD value was an indication of variation created. Highest interline variation, according to the most of the investigated traits was for PH, followed by EXE, whereas lowest variability was established for LW. Maximum variance observed for PH ranged from 116.17 for SA to 437.77 for SC, indicating that the variability for PH is spread around the mean value and from each other. Whereas, the small variance indicate that the LW tend to be very close to the mean and hence from each other. Statistical analysis revealed that the traits PH and EXE were diverged among 5 populations, indicating that there was good amount of variability for this particular trait. The interline variability comes from a genetic diversity of microspores which is an effect of random gene segregation during meiosis (Gemesne et al., 2001) and our results agree with this finding.

In our study, a significant and high r^2 value was obtained for individual trait among replications. The high r^2 values indicates that low variability or high uniformity of traits PH, TPL, LL, EXE III, EXE II, EXE I, EXE I whereas LW with low r^2 value was indication of highly variable or less uniformity of the particular trait. Scattered plot revealed that the values of individual trait were lying near the scattered point, indicating that the individual DH_1 lines/clones performed similarly at both replications without any trait segregation. However, the slight variation on the trait might be due to the influence of some environmental factors or lack of soil uniformity. The correlation study indicates a predictive relationship between the different phenotypic variable of DHLs within the population. Correlation coefficient (r) ranges from –1.0 to + 1.0. The positive r value signified the direct relationship, whereas negative value indicates the inverse co-relation between the two traits. PH was positive and highly correlated with TPL, INT II, INT I and EXE, whereas moderately associated with INT III and weakly correlated with LL. This implies that all above traits are related to each others. However, r values (-0.24 and -0. 20) signified that PN and LW were inversely correlated to PH. These results indicated that with increased in PH, other traits such as TPL, INT II, INT I, EXE and INT III were increased, whereas PN and LW decreased. PN was weak and negatively correlated with other traits. Again TPL was highly linked to INT (III, II, and I) and EXE.

The result of correlation study was evident from the comparative assessment of *EUI* and non *EUI* plants, depicted in Table 7. The comparative study indicates that in almost all lines, the *EUI* positive plant was taller compared to non *EUI* plant (Figure 3). Similarly, TPL is longer for *EUI* compared to non *EUI* plants. Elongation of Internode (INT III, INT II, INT I) and exertion of panicles (EXE) was higher in *EUI* compared to non *EUI* line (Figure 4). However, LW and PN was impact negatively. The variation among *EUI* and non *EUI* plants was mostly due to segregation of *eui* gene. Incomplete panicle exertion in most of the cytoplasmic male sterile (CMS) lines is one of the major impediments in obtaining higher seed yield, as 30–40% of the panicles are enclosed in the flag leaf and the enclosed spikelets are not available for cross pollination, thus resulting in lower seed yield (Gangashetti et al., 2004) and *EUI* trait has the potential to improves the panicle exertion through elongation of uppermost internode, which in turn increase seed yield. *EUI* is governed by recessive gene located in chromosome 5 (Gangashetti et al., 2006). The *EUI* trait being recessive in nature, its transfer into other varieties through conventional approach is cumbersome and time consuming, availability of doubled haploidy and molecular markers linked to *eui* gene can facilitate its introgression into desired genotype more effectively and efficiently. DH lines with *EUI* trait were found to be exhibit normal and elongated internode.

The SSR analysis thus revealed a sufficient degree of uniformity and purity of 247 DHLsout of 249 DH_0 plants. The absence of the heterozygous band indicates that all of these plants were homozygous at molecular level and they were

not of somatic cell origin. The results of the present study were also in accord to previous investigation (Sasmita, 2009). The degree of molecular uniformity in the present study indicates that first generation androgenic plant material can be efficiently used in the breeding program. However, to resolve the actual uniformity and polymorphism and relationship among the DHLs more primer pairs needed to be tested. Further, the foreground screening revealed segregation distortion of *eui* gene. Theoretically, the segregation must be in 1:1 ratio for *EUI*/non *EUI.* The probability of obtaining a desired genotype in doubled haploidy (1/2n) is much higher than in conventionally (1/4n) where n= number of genes controlling a particular character. However, in the present investigation *EUI* plants (152) out number non *EUI* plants (71). The deviation from the expected ratio might be due to death of DH plants during acclimatization, haploid plant regeneration or genotypic difference in anther response. In contrary to the result of trait segregation in the present investigation, Mandal and Gupta (1997) reported 1: 1 segregation patterns for some quantitative characters. Further, the expression of *EUI* trait in the first generation in the present results is indicates that recessive gene can be expressed instantly in the next generation.

The identified two DHLs showed hybrid banding pattern, consequence of retaining heterozygous loci in the genome and probably obtained from anther wall, not by androgenesis. The injuries to anthers during excision should be avoided in order to prevent somatic callus production from anther wall tissues (Reinert and Bajaj, 1977). Even *EUI* trait was not expressed in those lines, the reason seems to be due to recessive nature of *eui* which cannot express in heterozygous conditions. Evaluation of DH lines at molecular and field levels according to morphological traits gave a reliable answer about their uniformity and variability. The results of the present study indicate that the first generation androgenic rice lines are indeed doubled haploid lines or pure lines. The results are in accordance with previous investigations (Sasmita, 2009; Herawati et al., 2008).

CONCLUSIONS

In conclusion, the present investigation confirmed the homozygosity of first generation anther derived spontaneous diploids as doubled haploids. Doubled haploidy is a very useful tool for producing homozygous rice lines in a relatively short time.

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